Universidade de Lisboa

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Resumo

O crescimento dos tecidos é um dos processos fundamentais que contribuem para o desenvolvimento embrionário, no entanto, e apesar da sua importância, pouco se sabe acerca dos mecanismos que determinam o tamanho dos órgãos e consequentemente do organismo, uma das questões clássicas da Biologia do Desenvolvimento. Em particular, como é que as células desencadeiam os mecanismos adequados que asseguram as proporções certas e que resultam num tamanho final determinado com precisão? O controlo do tamanho dos tecidos/órgãos é um processo altamente coordenado e complexo que envolve diferentes mecanismos em resposta a sinais fisiológicos, incluindo factores circulantes como por exemplo hormonas e factores de crescimento semelhantes à insulina. Estudos clássicos sugeriram pela primeira vez, através de experiências de transplantação em membros de salamandra, que os órgãos podem possuir informação intrínseca sobre o seu tamanho final, mas o mecanismo subjacente continua por identificar. Recentemente, foi descoberta a via de sinalização Hippo, uma cascata de cinases que culmina na regulação de um potente regulador do crescimento. Por isso, esta via tem-se vindo a afirmar como um dos mecanismos regulatórios mais pertinentes na abordagem da questão do controlo do crescimento.

A via Hippo foi descoberta inicialmente em *Drosophila melanogaster* como uma potente cascata de fosforilação que envolve simultaneamente a coordenação da proliferação celular e da apoptose durante o desenvolvimento. Posteriormente, foi demonstrado que existem vários homólogos directos dos seus componentes em mamíferos e que a via assume um papel semelhante, indicando que está altamente conservada ao longo da evolução e que pode funcionar como um mecanismo global de regulação do crescimento em *Drosophila* e em vertebrados. Os primeiros componentes desta via foram identificados em rastreios genéticos em *Drosophila* que tinham como objectivo encontrar genes supressores de tumores, aqueles que quando mutados com perda de função induzissem um crescimento exagerado dos tecidos. Os primeiros genes a serem identificados foram *warts (wts)* e *salvador (sav)*, dois genes supressores de tumores, cujas proteínas interagem entre si. Foi demonstrado que a perda de função de qualquer um destes genes resulta num aumento da proliferação celular e redução da apoptose, o que constituiu a primeira evidência de regulação destes processos por estes componentes. Um passo decisivo na descoberta desta via de sinalização foi a identificação do gene *hippo (hpo),* o terceiro componente em *Drosophila.* Este gene, e consequentemente a via, acabaram por ser chamados de *hippo* uma vez que quando está mutado, o seu fenótipo é caracterizado por várias dobras de tecido com crescimento excessivo, fazendo lembrar as dobras na pele do hipopótamo.

Até agora, já foi identificado um grande número de componentes da via, tanto em *Drosophila* como em mamíferos, fazendo emergir uma complexa rede de reguladores positivos e negativos, que foi dividida em três grandes grupos: os componentes centrais e os componentes a montante e a jusante dos componentes centrais. Em *Drosophila*, o grupo dos componentes centrais é formado pelas cinases Hpo e Wts, e pelas proteínas adaptadoras Sav e Mats, que interagem entre si, estabelecendo uma cascata de fosforilação. Quando a via é activada, a cascata de fosforilação inibe o co-activador de transcrição Yorkie (Yki), que quando fosforilado, fica retido no citoplasma sem conseguir migrar para o núcleo e activar a transcrição dos seus genes-alvo. Por outro lado, quando a via está inactiva, Yki não é fosforilado e é transferido para o núcleo, onde se liga a diferentes factores de transcrição, promovendo a expressão de genes específicos, que induzem a proliferação celular e inibem a apoptose. Desta forma, a sobre-expressão de *yki* induz um sobre-crescimento nos tecidos, mediado pela transcrição activa dos seus genes-alvo, o que faz com que este gene actue como um oncogene, enquanto os outros membros nucleares actuam como supressores de tumores, inibindo a sua actividade. Em mamíferos, foi demonstrado que todos os componentes nucleares da via identificados na mosca têm homólogos directos como Mst1/2 (homólogos de Hpo), Sav1 (homólogo de Sav), Lats 1/2 (homólogos de Wts); MOBKL1 A/B (colectivamente denominado Mob; homólogos de Mats) e dois co-activadores da transcrição YAP e o seu parálogo TAZ (homólogos de Yki), e que interagem entre si da mesma forma descrita anteriormente em *Drosophila*.

O Peixe-zebra (*Danio rerio*), é um organismo frequentemente usado como modelo vertebrado para o estudo do desenvolvimento, uma vez que possui diversas características atractivas, como o rápido desenvolvimento, fácil reprodução e manutenção, e oferece várias possibilidades em termos de ferramentas genéticas, como por exemplo a criação de linhas transgénicas. Adicionalmente, o peixe-zebra é um modelo muito usado no estudo da regeneração pois, à semelhança de outros vertebrados inferiores, apresenta um grande potencial regenerativo, na medida em que consegue regenerar um elevado número de tecidos, tal como a retina, o coração, a espinal medula, as escamas e todas as barbatanas. Em particular, a barbatana caudal é uma das estruturas de eleição para o estudo da regeneração, pois pode ser facilmente acedida para efectuar uma amputação e danificada sem comprometer a sobrevivência do animal. Além disso, após amputação, a barbatana caudal tem a capacidade de recuperar o seu tamanho entre 7 a 14 dias. É de salientar, que independentemente do número de amputações ou outros factores externos, a barbatana recupera sempre o mesmo tamanho original, o que sugere a existência de um mecanismo altamente controlado que determina o tamanho correcto da estrutura a regenerar. Tendo em conta o curto espaço de tempo que este processo regenerativo necessita para ficar concluído, existe um aumento significativo da proliferação celular, mas que ocorre de forma controlada. Um dos factores que determina essa taxa de proliferação diz

respeito ao local de amputação, sendo que quanto mais proximal for o tecido amputado, ou seja, quanto mais tecido for amputado, maior será a taxa de proliferação e consequentemente mais rápida será a regeneração do tecido. Esta propriedade reflecte um mecanismo ao qual se chama memória posicional, que permite ao organismo reconhecer e regenerar apenas as estruturas/tecidos removidos na amputação.

Dadas as potencialidades da via Hippo ao nível da regulação do crescimento, esta apresenta-se como um bom candidato para abordar o estudo da regeneração no peixe-zebra. A sua grande conservação entre espécies já demonstrada nos diferentes modelos, faria prever que a via estivesse também presente no peixe-zebra, sendo este evolutivamente mais próximo dos mamíferos do que da mosca. Até agora, já foi evidenciado que alguns componentes tais como Mats, YAP e Lats estão conservados, no entanto, pouco se sabe ainda sobre as funções fisiológicas desta via neste organismo. Para mais conclusões, será necessário efectuar uma análise mais completa.

Tendo em conta todos estes dados, este projecto tem como principal objectivo ajudar a perceber qual o mecanismo que regula o processo de regeneração na barbatana caudal do peixe-zebra, que permite à barbatana recuperar sempre o seu tamanho original. Para tentar responder a esta questão, uma das mais antigas na área da Biologia Regenerativa, fomos estudar o possível envolvimento da via Hippo na regulação do tamanho final durante a regeneração. Nesse sentido, foi efectuada uma análise que abrange diversos componentes da via, através do estudo da expressão génica por hibridação *in situ* e RT-PCR não quantitativo. Esta análise permitiu-nos observar que vários componentes da via estão efectivamente a ser expressos durante a regeneração e os nossos resultados sugerem que a via Hippo pode desempenhar um papel importante na regulação deste processo de reparação. A via Hippo pode mesmo ser o regulador central que coordena o processo de crescimento da barbatana durante a regeneração, fornecendo às células sinais de proliferação ou apoptose, permitindo uma constante manutenção do tamanho da barbatana após amputação.

Palavras-chave: Via Hippo, controlo do crescimento, tamanho final, peixe-zebra, regeneração, barbatana caudal

Abstract

The Hippo pathway is a recently discovered signaling pathway that has been shown to be a potent growth regulator, whereas its deregulation leads to dramatic tissue overgrowth. This pathway is highly conserved throughout evolution, since its components are present and have direct homology between *Drosophila* and mammals, indicating this pathway as a potential universal mechanism of growth control. This topic is of outmost interest since the mechanisms by which the organism controls growth to obtain its final size remain as a long-standing question in Developmental Biology.

Zebrafish (*Danio rerio*), a vertebrate model frequently used in the study of development due to its several advantages such as the possibility of generating transgenic lines, has direct homologs to some of the components of the Hippo pathway, although the conservation of their function has not been shown. Additionally, zebrafish has a great capacity to regenerate several structures, such as the fins. The caudal fin is typically used to assess regeneration, since upon amputation it fully recovers the lost appendage with full functionality and restoring the original size, after 7-14 days. The original and final size of the fin is always restored regardless of the number of times it is amputated, which suggests the existence of a strict mechanism regulating growth during this repair process. Therefore, the Hippo pathway seems to be a promising approach to help understanding how the growth and final size are regulated during epimorphic regeneration.

In this work, we perform a broad and systematic analysis of several components of the pathway and show that most of them are effectively expressed in the fin during regeneration. Our data thus suggest that the Hippo pathway might be one of the key regulators of size maintenance upon injury, instructing the cells with proliferation or apoptotic cues, a long-lasting question in the field of Regenerative Biology.

Keywords: Hippo pathway, growth control, organ size, Zebrafish, Regeneration, caudal fin

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Introduction

1. The Hippo signaling pathway

Growth is a fundamental and universal process of animal development but the mechanisms that determine the control of organ size, and subsequently organism size, remain poorly understood. In particular, how do the cells trigger the proper mechanisms to ensure the right proportions resulting in an accurate final size? Organ size control is a highly coordinated and complex process that involves several mechanisms in response to physiological cues. These cues include circulating factors such as hormones and insulin-like growth factors (IGF) that are important to induce organ size[1]. Classical studies, through transplantation experiments in salamander limbs, were the first to suggest that organs might possess intrinsic information about their final size, although the underlying mechanism remains to be identified[1,2]. The recently discovered Hippo pathway emerged as a promising starting point to address this issue, and was shown, during the past decade, to be one of the key regulators of tissue growth[2-4]. It was first discovered in *Drosophila melanogaster* as a potent kinase cascade involved in the coordination of both cell proliferation and apoptosis during development[5,6] and was later shown to play a similar role in mammals[7-9]. Thus, the pathway is highly conserved throughout evolution, with many direct homologs between species, suggesting that it might work as a global regulator of organ size in *Drosophila* and vertebrates[2,3,10,11]. However, despite the extensive evolutionary conservation of Hippo signaling, there are some interspecific differences in the pathway components and the pathway appears to be more complex in mammals.

The first components of the pathway were identified in *Drosophila* genetic screens for tumor suppressor genes, on the basis that their loss-of-function led to dramatic tissue growth. These studies took advantage of some recent techniques allowing the generation of mosaics, enabling the analysis of homozygous clones in a heterozygous environment[2,3,6,10]. The first gene to be isolated, in 1995, using these mosaic-based screens was the tumor suppressor *warts (wts)* that is translated into a kinase[12,13]. Mutation of this gene results in a robust cell-autonomous overgrowth in different epithelial structures like the wings, the legs and the eyes[6] (Fig.1). In 2002, a second component was discovered after the identification of another tumor suppressor gene called *salvador (sav)*[14,15]. *sav* encodes a protein that was shown to interact directly with Wts and its mutations show a similar, but weaker, phenotype than *wts* mutations. In particular, it was demonstrated that loss of *wts* or *sav* increases cell proliferation and reduces apoptosis, providing the first evidence of regulation of these processes by these two components[6] (Fig.1). A major breakthrough that marked the beginning of the understanding of these proteins as components of a signaling pathway, was the identification of *hippo (hpo)* in *Drosophila*[16-20]. This gene also encodes a kinase which has been demonstrated to interact with Sav and Wts,

establishing a new kinase cascade[6]. This third tumor-suppressor gene, and consequently the pathway, was named Hippo because its mutant phenotype is characterized by several folds of overgrown tissues, resembling hippopotamus skin folds[3,21] (Fig.1).

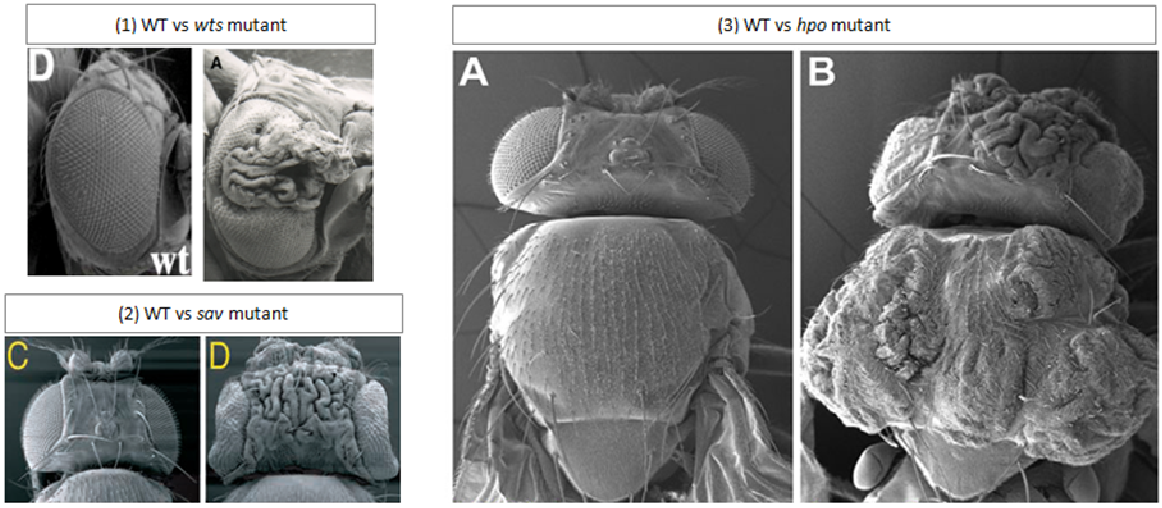

Figure 1 – Scanning electron micrographs of WT flies comparing with *wts* mutant(1), *sav* mutant(2) and *hpo* mutant(3). Figures from Xu *et al.* 1995([13]), Kango-Singh *et al.* 2002([14]), Tapon *et al.*2002([15]) and Halder *et al.* 2011([3]).

A growing number of genes have since then been identified and the respective proteins added as components of the pathway in *Drosophila* and other models. Consequently, a complex network including both positive and negative regulators has emerged and the pathway has been divided into core, upstream and downstream components.

In *Drosophila*, the core of the Hippo pathway includes a kinase cascade that ultimately results in the inhibition of the transcriptional coactivator Yorkie (Yki)[2,3] (Fig.2a). Yki has been identified as the missing link that researchers were looking for, to establish the connection between the core proteins and the target genes, being responsible for the regulation of the pathway transcription output[22]. Furthermore, overexpression of *yki* results in tissue overgrowth with increased proliferation and defective apoptosis, phenocopying the loss of the other pathway components. Thus, *yki* works as an oncogene, encoding a growth-promoter protein, while Hpo, Sav, Wts and Mats act as tumor suppressors by suppressing its activity[22]. Through biochemical studies it has been shown that when the pathway is activated, the Hpo kinase forms a complex with the adaptor protein Sav and together they phosphorylate another complex formed by the Wts kinase and a second adaptor protein called Mob as tumor suppressor (Mats)[23]. Upon phosphorylation, Wts-Mats complex phosphorylates and inhibits Yki, which gets retained in the cytoplasm, unable to migrate to the nucleus and induce the transcription of its target genes (Fig.2a). On the other hand, when the pathway is inactivated, Yki is not phosphorylated and is free to translocate to the nucleus, where it binds to different transcription factors, promoting the expression of specific target genes.

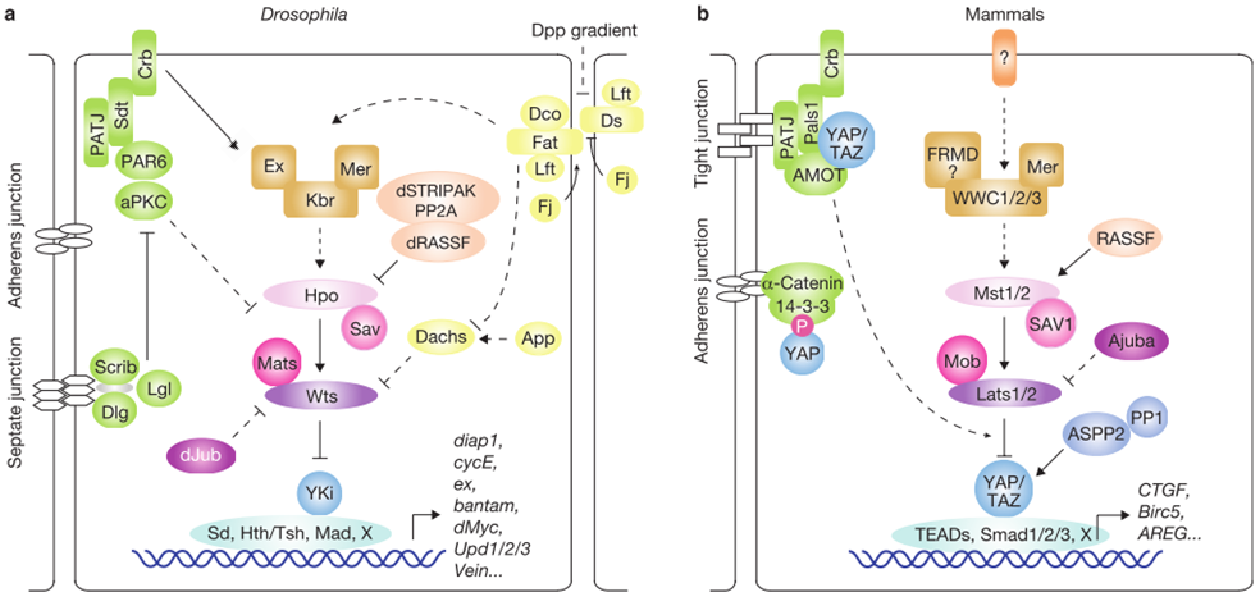

Figure 2 - A schematic view of the current understanding of the Hippo Signaling Pathway in *Drosophila* (a) and mammals (b). Equivalent proteins are indicated by matching colours. Arrowed or blunted ends indicate activation or inhibition, respectively. Dashed lines indicate unknown mechanisms. Adapted from Zhao *et al*. 2011([2]).

All the fly core components have direct homologs in mammals. The mammalian genome encodes Mst1/2 (Hpo homologs), Sav1 (Sav homolog), Lats 1/2 (Wts homologs); MOBKL1 A/B (collectively referred to as Mob; Mats homologs) and two transcriptional coactivators called YAP, Yes-associated protein, and its paralog TAZ (Yki homologs) (Fig.2b)[2,3,24]. The core kinase cassette in the mammalian pathway acts in a similar way, where Mst 1/2 and Lats 1/2, associated with the adaptor proteins Sav1 and Mob form a kinase cascade that leads to phosphorylation and inhibition of YAP/TAZ. As in the fly, if the transcription coactivator is inhibited, it cannot migrate to the nucleus and activate transcription of its target genes.

All together, the target genes and the binding factors that interact with Yki and YAP/TAZ are generically called the downstream members of the pathway. Since neither Yki nor YAP bind to DNA alone, the interaction with the transcription factors becomes essential to mediate their functions. In *Drosophila,* the primary transcription factor which plays a major role in interacting with Yki is the TEAD family transcription factor Scalloped (Sd), but other Yki partners which stimulate the expression of different target genes are also known, such as Homothorax (Hth), Teashirt (Tsh), and Mad[2,3,10,11,24]. Depending on the binding partner involved in the interaction, specific target genes which inhibit apoptosis (such as *diap1*) and others that induce proliferation (such as *bantam* and *cycE*) are activated. Activated Yki also increases the expression of several genes which encode components that will act upstream of the core proteins, such as *four-jointed (fj)*, *kibra*, and *expanded (ex),* creating a negative feedback loop[2,3]. In particular, *ex* is one of the genes that is regularly used as a reliable target gene to assess activation of Yki. Moreover, Yki

regulates the expression of genes that integrate other signaling pathways such as Notch, EGFR and Wingless[4,24].

The downstream components of the pathway are also highly conserved in mammals so, when there is no Hippo signaling, YAP/TAZ can enter in the nucleus and bind to TEAD 1/4 (Sd homologs)[25] or other transcription factors. Once bound to these specific interaction partners, they will activate the transcription of their targets, in particular proliferation and anti-apoptotic genes, like in *Drosophila*. It is presently unknown if a negative feedback loop like the one described in *Drosophila* is conserved in mammals, and if the orthologs of Kibra or Ex (Frmd6) conserve the role of target genes of the pathway. One of the transcriptional targets of YAP/TAZ is the *connective tissue growth factor* (*ctgf*), which encodes a cytokine important for cell proliferation and cell growth[25,26]. CTGF was shown to be crucial for YAP-induced proliferation and anchorage-independent growth, because when the gene is knocked down, it partially impairs the oncogenic potential of YAP[25]. Another downstream target that contributes to YAP-mediated cell proliferation is *amphiregulin* (*areg*), as its knockdown inhibits the proliferative effects of YAP[27]. This gene encodes the ligand of the epidermal growth factor receptor (EGFR), which indicates that interaction between Hippo and other highly conserved pathways is also present in mammals. Regarding the anti-apoptotic genes, an example of a direct target of YAP/TAZ is *birc5,* a homolog of *Drosophila diap1*. This gene encodes a member of the inhibitor of apoptosis protein (IAP) family which is also a mitotic regulator[8].

When *yap* is overexpressed in adult mouse livers, the result is a dramatic increase in organ size due to cell proliferation[7,8], but when the overexpression ceases, livers return to the normal size, suggesting the activation of a size-control mechanism through Hippo signaling, probably inducing apoptosis to reduce the number of cells[7]. Surprisingly, it has been reported that besides *yap* being a potent oncogene, YAP can also bind to the tumor-supressor p73[28], a p53 family pro-apoptotic transcription factor, thereby activating the transcription of specific target genes known to induce programmed cell death. There are contradicting reports addressing this issue, hence it is still unclear how YAP is induced to trigger apoptosis, by p73-binding, and to repress TEAD 1/4-mediated apoptosis, in distinct situations[2,3,24]. Post-translational modifications of YAP dependent of the context might induce YAP to choose selectively between the different transcription factors[3]. In fact, Hippo signaling shows multiple context-dependent outputs and there are at least two mechanisms that regulate and contribute to these different outcomes. One is the large range of binding factors that interact with the specific transcriptional coactivator, and the second is that the transcriptional output of the pathway is cell-type dependent.

While the core and the downstream components of the pathway are relatively well established, the same is not verified for the upstream interacting proteins and mechanisms that regulate Hippo signaling. Recent studies have addressed this question and revealed that many

different proteins act upstream of the core kinase cascade[2,3,24]. One of the first studies on this topic, in *Drosophila,* revealed that Merlin (Mer) and Ex, two tumor suppressors, act upstream and through Wts activation, having partially redundant functions[29]. In addition, Kibra interacts with these two components, forming a Ex-Mer-Kibra complex that is apically localized in the cell and is able to regulate the core kinase cascade through different protein-protein interactions, including Hpo activation[3,10,24]. Together, these proteins constitute the upstream components of the pathway. In vertebrates, these upstream components and their regulation mechanisms of the kinase cascade mediated by Mer/NF2[9], Kibra and Frmd6 (Ex ortholog)[30] are conserved.

In growth control regulation, the information on organ size must be transmitted to single cells, leading them to make decisions. Recent studies have shed some light on how the pathway is regulated, showing multiple inputs that feed into the pathway forming a very dynamic and complex network. These various inputs can respond to developmental cues or stress signals and they can act at different levels of the kinase cascade, independently or in a coordinated-manner. For instance, in *Drosophila*, cell-cell interactions have an impact in the Hippo pathway and the transmembrane protocadherin Fat was identified as an important player in this upstream regulation of the pathway[2,3]. Fat can activate Hpo signaling through two independent mechanisms: increasing the levels of apical membrane-localized Ex, along with Mer and Kibra, which induces Hpo and Wts activation, or inhibiting the Dachs myosin that mediates Wts degradation[2,3,24]. Furthermore, Fat interacts with its ligand Dachsous (Ds), which is another cell-surface protocadherin, and this interaction may be regulated by several other components, such as the kinase Four-jointed (Fj) which modulates their activity by phosphorylation of their extracellular domains. Also proteins involved in the determination of cell polarity have been implicated in Hippo pathway modulation, such as the transmembrane apical determinant Crumbs (Crb) that forms a complex with several other proteins, contributing for the maintenance of apical-basal cell polarity[2,3]. Crb also seems to be important for the apical localization of Ex, inducing the Hippo signaling, indicating a role of cell polarity in the regulation of the Hippo pathway. Recently, dJub, a protein that interacts directly with Wts and Sav, was shown to negatively regulate Hippo signalling, but the detailed mechanism remains to be revealed[31]. Additionally, Hpo can be inactivated by direct dephosphorylation mediated by a complex that includes the protein phosphatase 2A (PP2A) and the Striatin-interacting phosphatase and kinase (STRIPAK)[32] (Fig.2a). This complex seems to be recruited by *Drosophila* Ras association domain family (dRASSF) that may compete with Sav for Hpo binding.

In vertebrates, regulatory roles of AJUB (dJub homolog) and RASSF (dRASSF homolog) are conserved, although in this case RASSF works as an activator of Mst1/2 (Hpo homolog) (Fig.2b). Some studies indicate that the mammalian homologs of Fat and Crb may also be important for the regulation of this pathway, but it is still unclear. Other inputs than those described for

Drosophila are known in mammals, such as cell density, which cells sense through different mechanisms. One of these mechanisms is cell morphology, which has been recently shown as an important factor in the regulation of the Hippo pathway[33]. For instance, when cells are flat and spread, this morphology induces the formation of stress fibers (F-actin), which regulate YAP subcellular localization through interactions with the kinase cascade[33]. Additionally, it has been demonstrated that the stiffness of the extracellular matrix (ECM), which cells sense through stress fibers and cytoskeleton tension, also regulates YAP/TAZ activity, although this work reported that the mechanism acts in parallel and independently of the Hippo pathway[34]. Another mechanism by which cells sense density is cell adhesion, in which α-catenin, an adherens junction protein, was recently shown to be implicated. α-catenin acts as a tumor suppressor and upstream regulator of YAP, sequestering it in the cytoplasm and thereby inhibiting its activity[35,36]. Therefore, this indicates that cell adhesion may be important in the regulation of the Hippo pathway. This cytoplasm retention, mediated by α-catenin through the modulation of YAP interaction with the 14-3-3 binding protein, keeps this complex next to the adherens junctions (Fig.2b) and prevents dephosphorylation by the PP2A phosphatase. Moreover, regarding cell contact and adhesion, Mer/NF2 which has been linked to the mammalian Hippo signaling as an upstream regulator[9,37], works as a tumor suppressor by controlling the cell-cell interactions mediated by cadherins[38]. In particular, the transmembrane receptor CD44 is necessary for Mer growth-inhibitory activity and can mediate cell contact inhibition through ECM cues, exclusively in mammals[39]. In addition, Mer has also been reported to be associated with the tight-junction-associated protein complex that comprises the proteins Angiomotin (Amot), Patj, and Pals1[40] (Fig.3b), supporting the hypothesis that cell adhesion is involved in the regulation of the Hippo pathway. It is worth noting that most of these studies have been performed in cell-culture, so further experiments are needed to test if the pathway is modulated in the same ways *in vivo*.

Activation of Hippo signaling triggers the phosphorylation and subsequent inhibition of YAP, TAZ and Yki transcriptional coactivators. Several mechanisms have been described, in both systems, where these effector proteins can be directly inhibited. In *Drosophila*, the regular mechanism of Yki inhibition involves phosphorylation of three different sites: Ser 111, Ser 168 and Ser 250[3,10]. However, the Ser 168 residue is particularly relevant since its phosphorylation creates a binding site for a 14-3-3 binding protein. This protein will bind to Yki and retain it in the cytoplasm, suppressing its transcriptional activity[2] (Fig.3a). The same mechanism is conserved in the mammalian pathway, where the corresponding phosphorylation sites are Ser 127 for YAP and Ser 89 for TAZ[2,3,8]. In mammals, another mechanism of YAP inhibition, which involves a specific phosphorylation of the Ser 381 residue, can lead to YAP/TAZ degradation (Fig.3c). This phosphorylation involves Lats 1/2 and casein kinase 1 (CK1δ/ε), which induces a ubiquitylation mediated by the SCFβ–TRCP complex and subsequent proteosomal

degradation[2,24]. Furthermore, other mechanisms that inhibit Yki/YAP in a phosphorylation-independent manner have been described. For instance, Yki can get sequestered in the cytoplasm, by physical interactions through WW domain–PPXY motif bonds, with Hpo, Wts and Ex[2,3] (Fig.3b), while in the case of YAP/TAZ, a similar physical inhibition mechanism was recently shown to be performed by Amot family proteins which are part of a tight-junction protein complex[41,42].

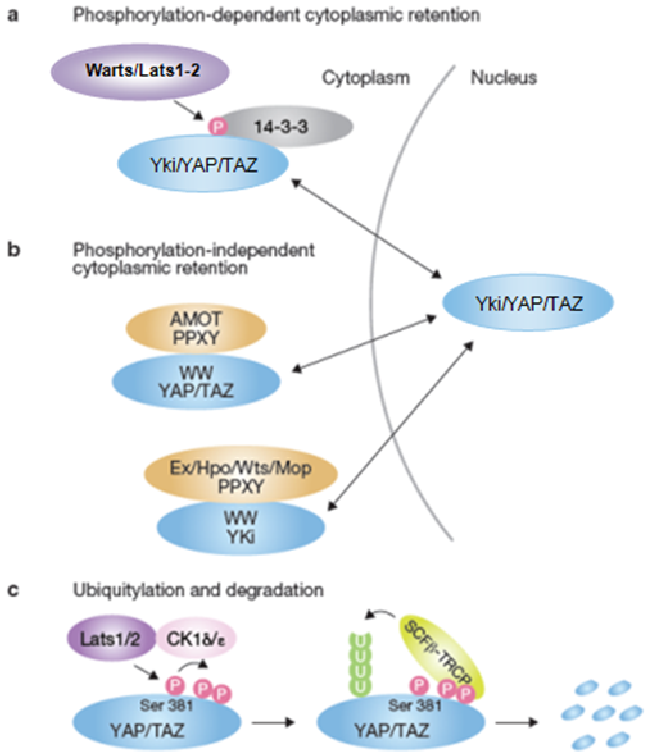

Figure 3 - Mechanisms of Yki/YAP/TAZ inhibition by the Hippo pathway. (a) Phosphorylation-dependent cytoplasmic retention; (b) Phosphorylation-independent cytoplasmic retention; (c) Phosphorylation-induced ubiquitylation and degradation. Adapted from Zhao *et al*. 2011([2]).

2. Zebrafish as a model organism to study the Hippo pathway

For decades, zebrafish has been extensively used as a model organism to study many different topics. It is a particularly good model to study developmental biology, mainly because it is a vertebrate with external fertilization, transparent embryos and a fast development process, which allows easy study of all stages of the development. Several other features of this organism such as the high regenerative potential and the fully sequenced genome made it a very popular model system to work with. The sequencing of the genome was of great importance since it enabled the development of multiple genetic tools, such as genetic screens and the generation of

transgenic lines. Furthermore, the small size enables the maintenance of large numbers of individuals.

The Hippo signaling pathway has been extensively studied in *Drosophila* and mammals, while in zebrafish, which is evolutionarily closer to mammals, little is known about its components and physiological functions. So far, orthologs of *fat*[43], *lats1-2*[44], *mob*[45], *yap*[43,46] have been identified.

In zebrafish, *mats* was the first component of the pathway to be reported and three orthologs were identified. Knockdown of one of these *mats* family genes (*mats1*) caused a global development delay, showing a requirement for growth control and normal embryonic development[45]. This phenotype is similar to those obtained in *Drosophila mats* homozygous mutants which indicates a general deregulation of the Hippo pathway. In mosaic flies, loss of function of *mats* results in localized tissue overgrowth only[23]. Notably, the same phenotype is observed in chimeric zebrafish embryos where the *mats1* morphant cells have a growth advantage and proliferate more than normal cells[45]. Thus, *mats1* seems to be playing a critical role in the regulation of cell proliferation and apoptosis during early development in zebrafish, thereby establishing the first link to the Hippo pathway in this model system. Moreover, this work shows the conservation of *mats* growth-inhibitory role between vertebrates and invertebrates.

In the case of zebrafish *yap (yap1)*, it was demonstrated through bioinformatics analysis, that it shares high identity with the ortholog in *Drosophila*[50]. Knocking down this gene in embryos, with a morpholino approach, causes several morphological defects such as reduced eyes and brain size as well as branchial arches with less cartilages[50], so *yap* seems to be required for fish embryogenesis, at least for the development of these structures. Moreover, *yap* overexpression can lead to cyst formation in pronephric development[43], indicating that the Hippo pathway is necessary for normal embryonic kidney development. Additionally, this work suggests a possible connection between cell polarity and the Hippo pathway conserved in vertebrates, in which Fat1 (Fat homolog) seems to be involved[43].

Regarding Lats, which represents a critical component of the core of the Hippo pathway, two paralogs have been described in zebrafish[44]. *lats1* is the direct homolog of the gene present in *Drosophila (wts)* and is the most functionally conserved, while *lats2* is the result of zebrafish genome duplication. A morpholino-mediated knockdown of both genes affects cell migration during gastrulation, suggesting that *lats* is important in gastrulation movements[44].

Furthermore, the orthologs of two mammalian target genes of the Hippo pathway have been described in zebrafish, namely ctgf[47-49] and *birc5/survivin*[43,50-52]. Birc5 is an inhibitor of apoptosis and knockdown of Fat1 (or Scribble) resulted in the upregulation of *birc5*[43], which indicates that it might work as a target of the Hippo pathway in zebrafish as well. Another target

of YAP described in mammals is *ctgf* and in zebrafish there are also two homologs of this gene (*ctgf-c19* and *ctgf-c20*)[49]. They are expressed in several embryonic structures, such as somites and notochord, but its possible role as a target gene of the Hippo pathway was not assessed. Therefore, further experiments are necessary to confirm these putative target genes as actual targets of YAP, and subsequently, the pathway.

These recent reports highlight that several of the main components of the Hippo pathway, already shown to be conserved between *Drosophila* and mammals, are also present in zebrafish. They also suggest that the pathway maintains its functions in growth-control, however, most of these studies describe only expression patterns of single genes and do not analyze them in a signaling pathway context so a more complete characterization of the pathway in zebrafish is of high interest. Furthermore, since the zebrafish model offers many genetic tools, including the generation of transgenics, which allow manipulation of the system *in vivo,* it is a good model organism to study the Hippo signaling pathway and its underlying regulation mechanisms. Additionally, since all of this work has been reported in early stages of the development, it would also be interesting to study the Hippo pathway in the context of the adult fish.

3. Regeneration in zebrafish and possible connection with the Hippo pathway

Regeneration can be defined as the reconstitution of a lost or injured tissue. This is a broad concept which covers several different phenomena such as physiological regeneration, morphallaxis, hypertrophy and reparative regeneration. Physiological regeneration includes physiological processes such as the replacement of blood and epithelial cells or the hormonal cycles. Morphallaxis is defined as the reconstruction of the organism form by remodeling the body after severe damage (e.g. hydra). Hypertrophy is associated with the compensatory increase or restoration of the mass of an internal organ (e.g. kidneys and liver). Reparative regeneration includes repair and epimorphic regeneration[53] and consists in the process that leads to the complete reconstitution of an organ or tissue with multiple cell types, mediated by the formation of a blastema (e.g. amphibians and fish appendages)[54]. The blastema is a specialized structure which results from the proliferation of undifferentiated progenitor cells, followed by the differentiation of these progenitors, fully restoring the missing tissue[53,54]. Its origin has been a long-standing question in the field and, until recently, very little was known about how this structure is formed. Two hypotheses were proposed for the origin of the blastema: the first hypothesizes that the blastema derives from stem (or progenitor) cells, and the second states that it arises from dedifferentiation or transdifferentiation of mature cells around the wound site. Recently, it was shown that transdifferentiation between cell lineages does not happen in the regenerating fin[55] and that, in response to amputation, differentiated

bone cells called scleroblasts (corresponding to mammalian osteoblasts) acquire a proliferative state, migrate distally towards the blastema where they integrate and dedifferentiate, thereby contributing to blastema formation[56,57]. Consistently, these cells remain lineage restricted so, after forming the blastema, they only redifferentiate into osteoblasts[57]. These reports provide novel insights which favour the second hypothesis stated above. Further studies addressing the origin of the cells that mediate the epimorphic regeneration in zebrafish appendages, may translate into potential advances with application to human tissue repair.

Zebrafish retain a high potential for regeneration, particularly when compared with mammals, being able to regenerate a variety of tissues, including the fins, retina, lens, scales, heart and spinal cord. Unlike the situation in mammals, fish tissues with regenerative abilities never form scars after injury, since they undergo a complete tissue reconstitution process. All five fin types of zebrafish can regenerate, but the caudal fin is most frequently used for regeneration studies since it can be easily accessed for surgery, injured without compromising survival of the organism and it is a simple and symmetric structure[54,58]. The caudal fin is composed of several tissues: epidermis, blood vessels, nerves, connective tissue, pigment cells, lepidotrichia, which are multiple elongated bone rays with a dermal origin and actinotrichia that are bundles of collagen fibers that surround the bone rays at distal positions. Moreover, a lepidotrichium is composed of multiple segments of two hemirays joined by ligaments and runs from the proximal to the distal part of the fin where it can bifurcate[53,54,59].

The epimorphic regeneration in the caudal fin can be triggered by injury, such as an amputation, and is a very rapid process. It includes three main stages: wound healing, blastema formation and regenerative outgrowth[53,54] (Fig.4). The wound healing starts with the migration of a thin epidermal layer that will cover the wound within 3 hours post amputation (hpa), but the epidermal accumulation lasts until 18hpa, when a thick layer of epidermal cells, called wound epidermis, is finally formed. The wound epidermis is a main structure of regeneration and its formation is critical, since it has been shown that there is no regeneration process without it. By 18-24hpa, a basal epidermal layer composed of cuboidal cells starts to take shape and it plays an important role in the communication of growth and patterning signals, since it is the border between the wound epidermis and the forming blastema. Furthermore, this initial phase is characterized by the migration of epithelial cells, but does not involve cell proliferation. Following wound healing, the blastema is formed between 12 and 48 hpa. Blastema consists in a disorganization of the mesenchymal tissue, whose cells migrate distally from the amputation plane, dedifferentiate and proliferate, as recently demonstrated. Thus, the blastema which is the essential structure of the epimorphic regeneration consists of a proliferative mass of mesenchymal cells which lead to the formation of the new structures of the fin. By last, the regenerative outgrowth is initiated at 48hpa and in this stage the main events are the

proliferation, differentiation and patterning of the blastemal cells, so the lost structures can be replaced. This phase is also characterized by a reduction of cell cycle length, enabling the quick growth of the fin which takes between 7 and 14 days to restore its original appearance.

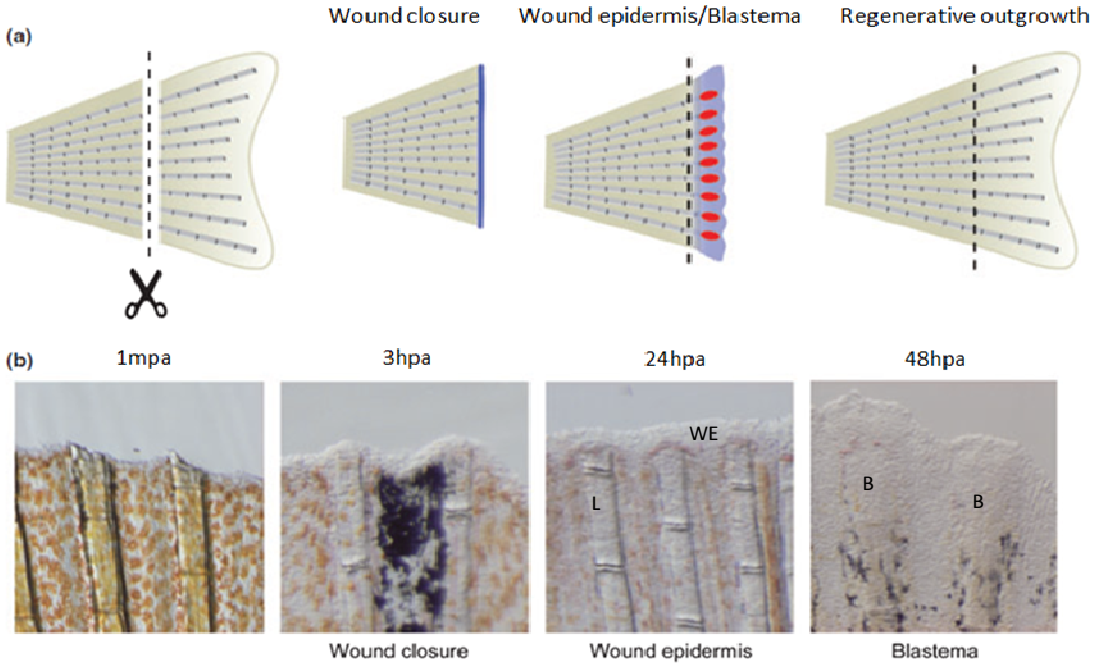

Figure 4 - Epimorphic regeneration in adult fish. Process of fin regeneration (a) and actual tissue appearances (b). mpa - minutes post amputation; hpa- hours post amputation. B:Blastema (Red ovals); L:Lepidotrichium; WE:Wound Epidermis. Adapted from Kawakami *et al*. 2010([53]).

There are many signaling pathways involved in development, many of which are simultaneously implicated in regeneration, such as Fibroblast Growth Factor (FGF), Wnt and Sonic hedgehog. In particular, a model has been proposed, in which FGF signaling mediates a position-dependent control of growth rate. This may be the mechanism giving instructions to the cell about the regeneration rate or the amount of tissue to regenerate, a phenomemon referred to as positional memory[60], which enables the organism to recognize and regenerate only the structures removed by amputation. It is believed that this positional information leads to the quick establishment of a gradient along the proximodistal (PD) axis, right after amputation, ensuring the recovery of the right structures after loss or damage, although the underlying molecular mechanism is still unclear.

The Hippo pathway has started to be studied in the context of regeneration, since it is a potent growth regulator coordinating cell proliferation and apoptosis, and therefore may play an important role in the regulation of this process. A recent study has reported a link between the Jun N-terminal Kinase (JNK) pathway, a damage-sensing pathway also activated in the early phases of regeneration[61], and the Hippo pathway in *Drosophila*[62]. This work shows that Hippo pathway components (Wts and Yki) coordinate the proliferation of intestinal stem cells (ISC), in

order to facilitate the repair of the intestine after damage[62]. In the differentiated cells, such as enterocytes, Yki is activated in response to tissue damage or activation of the JNK pathway, promoting an increase of the proliferation of ISC. This reveals a new role for the Hippo pathway components in regulating stem cell proliferation and intestinal repair, while it establishes a new link between the JNK and Hippo pathways, in which both are activated upon damage and JNK is upstream of Yki, in this repair process. Another study has shown that the Hippo pathway is activated in the mammalian intestine after damage[63]. While YAP is not necessary for normal intestinal homeostasis in mammals, it is absolutely required for tissue repair after injury, since it is necessary for cell proliferation. However, a tight control is necessary in order to promote cell proliferation and simultaneously inhibit excessive (potentially oncogenic) growth. So, these studies indicate the Hippo pathway may be a player in the repair process and reveal that this function is conserved between different species. Importantly, none of these repair models reflect epimorphic regeneration since there is no blastema formation, so the regeneration in the fish fin, in particular proliferation, apoptosis, restoration of the original fin size and its maintenance, might be regulated in a different way.

Overall, these data point the Hippo pathway as a likely candidate to have impact in the regulation of the fin size during epimorphic regeneration. To test this hypothesis, we studied the expression of several genes of the pathway in the regenerating fin of zebrafish, through *in situ* hybridizations and RT-PCR, with the objective of characterizing this system and to potentially understand better how the pathway works in zebrafish. The control of organ size is a fundamental feature of biology and is associated to some of the oldest questions of developmental biology, which remain unanswered. In particular, it is amazing how the regenerating fin of zebrafish always maintains the same original size, regardless of the age of the fish or the number of amputations, which suggests the existence of a potent and tightly regulated growth-control mechanism. With this project we aimed to provide new insights into the potential role of the Hippo pathway in regulating the final size of the zebrafish caudal fin during its regeneration.

Materials and Methods

1. Zebrafish husbandry, manipulation and amputation

Wild-type AB strain adult zebrafish, *Danio rerio,* used in the experiments were kept at 28ºC. For amputations, 3–6 months old adult fish were anesthetized in Tricaine 1x (160 mg/ml) (MS222, Sigma) and the caudal fins were amputated using a scalpel. Then, regeneration was allowed to proceed until desired time points, while keeping the fish in crossing boxes on a 33°C water bath, which is the standard temperature that allows a faster regeneration process. Fish were then anesthetized again to collect the regenerates for analysis.

Embryos were generated from crosses between Wild-type AB strain adult fish, in which single mating pairs were put together overnight in crossing boxes. They were collected the next day and kept at 28ºC in embryo medium 1x (from stock solution 50x 14,69g NaCl, 0,63g KCl, 2,43g $CaCl_2.2H_2O$, 4,07g $MgSO_4.7H_2O$ with 1ml methylene blue /10L of solution) until reaching the desired developmental stages.

2. Cloning

The genes *nf2b* and *birc5a* were cloned using the TA overhangs cloning strategy. For that, total RNA of 5 days post-fertilization (dpf) embryos was extracted as described in Chomczynski and Sacchi 1987([64]) using Trizol (Invitrogen) which is a *ready-to-use* monophasic solution of phenol and guanidine isothiocyanate. Following extraction, RNA was transcribed into total cDNA with the *1st Strand cDNA Synthesis Kit for RT-PCR (AMV)* (Roche), using oligo(dT) primers, according to the manufacturer's protocol. After the cDNA synthesis and using the respective pair of primers (Invitrogen) for each gene (Table 1), a PCR was made to amplify exclusively the gene of interest (*nf2b* or *survivin 1*). The PCR settings for *nf2b* were: 94ºC for 5 minutes, 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 1 minute repeating for 35 cycles, and 72ºC for 10 minutes; and for *survivin 1:* 94ºC for 5 minutes, 94ºC for 30 seconds, 58ºC for 1 minute, and 72ºC for 1 minute repeating for 35 cycles, 72ºC for 7 minutes. Amplification of the fragments was confirmed by electrophoresis and DNA was purified using the *PCR Clean-Up System Kit* (Promega). Following purification, the fragment of each gene was ligated to the vector *pGEM-T-easy* (Promega), using T4 DNA Ligase (Promega) overnight at 4ºC. In the case of *nf2b* cloning, after the PCR amplification, the fragment was extracted from the electrophoresis gel using the *QIAquick Gel Extraction Kit* (Qiagen), according to the manufacturer's protocol, and polyethylene glycol (PEG, 5%) (Fermentas) was used to increase the ligation efficiency. After ligation, the constructs were transformed into Top10 chemically competent cells and the transformed cells

were plated along with 100µl of IPTG (0,1M) (Sigma) and 20µl of X-Gal (50mg/ml of formamide) (Duchefa), which enables the selection of transformed cells which integrated the construct with the insert (white cells). Following selection, the correct ligation was confirmed with the restriction enzymes XhoI (Promega) for *nf2b* and MlsI (Fermentas) for *survivin 1*, which only cut a specific sequence inside the insert but not in the vector. This cloning method is not directional so cloned constructs were sequenced to determine the direction of insertion.

3. Semi-quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

3.1 RNA extraction and cDNA synthesis

For the RT-PCR analysis, the first bony ray segment and the blastema tissue of caudal fins from adult zebrafish of three regeneration stages (24, 48 and 72 hours post-amputation), were collected for RNA extraction (5 fins per stage). Also, uncut fins were collected for negative controls. Total RNA was extracted using Trizol (Invitrogen) as previously described. Additionally, RNA of 5dpf embryos was extracted to use as a positive control. After extraction, RNA was quantified in a nanodrop spectrophotometer and total cDNA was then generated from 1µg of total RNA from each sample, using the *1st Strand cDNA Synthesis Kit for RT-PCR (AMV)* (Roche) according to the manufacturer's protocol.

3.2 RT-PCR settings and primers

For the RT-PCR, we have standardized the conditions for every reaction of all the genes: 1µg of cDNA, primers at 10mM (Invitrogen), PCR Reaction Buffer (-$MgCl_2$) 1x (Invitrogen), dNTP 10mM (Invitrogen), DMSO 5% (Sigma), $MgCl_2$ 5mM (exception: 2mM for *β-actin*) (Invitrogen) and Taq DNA polymerase Platinum 5U (Invitrogen). The primers used are listed in Table 1. The PCR settings for *stk3, yap* and the controls *cmlc2* and *β-actin* were: 94ºC for 5 minutes, 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds repeating for 25 cycles, and 72ºC for 1 minute. The PCR settings for *ctgf*, *frmd6*, *nf2b* and the controls *cmlc2* and *β-actin* were: 94ºC for 5 minutes, 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds repeating 35 cycles, and 72ºC for 1 minute.

Gene	Forward Primer (5')	Reverse Primer (3')	Product Length
β-actin[1]	TTCACCACCACAGCCGAAAGA	TACCGCAAGATTCCATACCCA	223 bp
birc5a (survivin1)	CAACCTCCCACAAAATGGATCTTGC	ATGCTCTCAATGAACCTCTTCATTC	429 bp
cmlc2	TTTGGCTGCATAGATCAGAACC	TTTTTTCTGAGAGCAACTGAGTATGA	751 bp
ctgf-c20[2]	TCACCTGGTGTAAGCCTAGTTCTGG	GGCATGCGCAGGTCTTGATGAAC	847 bp
four-jointed	GCGGTGCAAAAAGTTTTAATATTC	ACAAATCTGGTACAGATTTTCTTCC	1026 bp
frmd6	CCATCGATATGAGCAAACTGACTTTCCACA	CGGGCCCTTACACCACAAACTCTGGTTCTG	1851 bp
nf2b	CGGGCCTGGTTTAACACATA	CAACAGAGCTCGGATTGTTCT	1831 bp
salvador1	GTGTCAGTGCCAACCTGGAT	AGTAAGCTGTCTGAGTGTGTCA	1302 bp
stk3	GCAGTGCTTCCTTAAACTCCAAAC	GCAGGAATCTAGAGTAAGATGCAG	1667 bp
yap1	CGACTTTCCTTGAAAACGGT	AAGGTGTAGTGCTGGGTTCG	1417 bp

Table 1 - List of primers used for cloning and semi-quantitative RT-PCR. [1]Primers published in Schebesta *et al.* 2006([65]). [2]Primers published in Fernando *et al*. 2010([49]).

4. *In situ* hybridization

4.1 RNA probes

DIG-labelled antisense RNA probes for some genes (*ctgf-c20*, *frmd6* and *nf2b*) were synthesized following the protocol described in Henrique *et al*. 1995([66]). The probes for the remaining genes studied (*c-jun*, *four-jointed, junb, sav1, stk3, surv1, surv2 and yap1*) were synthesized following a protocol adapted from Henrique *et al*. 1995([66]). The modifications were: after plasmid linearization, DNA was purified using the *PCR Clean-Up System Kit* (Promega). Then, 1µl of DNAse I (Roche) was added following transcription, for 15 minutes at 37ºC, to eliminate non-transcribed DNA, and the probe was denatured for 10 minutes at 70ºC. To purify the probes, *illustra ProbeQuant G-50 Micro Columns Kit* (GE Healthcare) was used and 50 µl of deionized formamide (Sigma) were added, to preserve the RNA. The correct sizes of all probes were confirmed by electrophoresis and all the probes (Table 2) were stored at -20ºC until needed.

Gene	Plasmid	Sense Probe		Antisense Probe		Hybridization Temperature
		Restriction Enzyme	Polymerase	Restriction Enzyme	Polymerase	
birc5a (survivin 1)	pGEM-T-Easy	ApaI	SP6	SalI	T7	66ºC
birc5b (survivin 2)[1]	pGEM-T	NotI	T7	SalI	T3	67ºC
c-jun	pGEM-T-Easy	NcoI	SP6	SalI	T7	67ºC
ctgf[2]	pSport	BamHI	T7	EcoRI	SP6	67ºC
four-jointed	pGEM-T-Easy	SalI	T7	NcoI	SP6	67ºC
frmd6	pCS2	EcoRV	SP6	BamHI	T3	68ºC
junb	pGEM-T-Easy	SalI	T7	NcoI	SP6	68ºC
nf2b	pGEM-T-Easy	NcoI	SP6	SpeI	T7	67ºC
salvador1	pGEM-T-Easy	NcoI	SP6	SalI	T7	66ºC
stk3	pGEM-T-Easy	ApaI	SP6	SalI	T7	67ºC
yap1	pGEM-T-Easy	SalI	T7	NcoI	SP6	68ºC

Table 2 - RNA probes, polymerases used for their transcription and plasmids in which the genes were cloned.[1]Plasmid kindly provided by Dr. Anskar Leung and published in Ma *et al.* 2009([51]).[2]Plasmid kindly provided by Dr. Uwe Strähle and published in Dickmeis *et al*. 2004([47]).

4.2 Whole-mount *in situ* hybridization

4.2.1 Embryos

We used the protocol of Thisse and Thisse 2008([67]) with the following modifications: embryos were manually dechorionated only after rehydration, using sharp forceps. Digestion with proteinase K was performed at 20 µg/ml in PBT. Embryos were hybridized in Hybridization Mix (HM) containing 5-10µl/ml digoxigenin(DIG)-labeled RNA probe, overnight at 65-70°C, depending on the probe (Table 2). For stages older than 24 hours post-fertilization (hpf), embryos were treated with 1-phenyl-2-thiourea (PTU, 0.03mg/mL) (Sigma), to prevent the formation of melanin pigment.

4.2.2 Adult caudal fins

We used a protocol adapted from Sousa *et al.* 2011([56]) with the following modifications: after fixation, fins were dehydrated in methanol (MeOH) at room temperature (RT) and stored at -20°C at least overnight. Fins were then rehydrated in a MeOH/phosphate buffered saline with 0,1% Tween20 (PBT) series. Embryos were pre-hybridized for at least 3 hours at 65-70°C in HM, the same used for the embryos protocol. Fins were hybridized in HM containing 10-15µL/ml DIG-labeled RNA probe, overnight at 65-70°C, depending on the probe (Table 2). In the second day, fins were incubated with blocking solution containing 10% goat serum in TRIS-buffered saline with 0,1% Tween20 (TBST) at RT for at least 3 hours. In the third day, fins were equilibrated (3x 5 minutes or more) at RT in staining buffer NTMT (5 M NaCl, 1 M Tris-HCl pH 9.5, 1 M MgCl2, 10% Tween 20, in H2O MQ), the same used for the embryo protocol.

Additionally, Levamisole hydrochloride (Sigma) was added to the NTMT buffer to reduce some of the background staining (0,02g levamisole/25ml of NTMT). The fins were developed using NBT/BCIP staining solution (Roche) in NTMT buffer (1µl/ml NBT, 3.5µl/ml BCIP).

4.3 *In situ* hybridization in longitudinal sections of adult caudal fins

This protocol was adapted from Smith *et al.* 2008([68]) and performed with the following modifications: after fixation and rehydration, fins were equilibrated in phosphate buffered saline (PBS) with 30% sucrose overnight at 4ºC and embedded in gelatin the next day. The fins embedded in gelatin were frozen, and the gelatin blocks were kept at -80ºC. The frozen blocks were subsequently sectioned (12 µm) using a cryostat (Leica) and sections were stored at -20ºC until needed. Frozen sections were thawed at RT during 30-60 minutes. Proteinase K (10 mg/ml in PBS) digestion was performed for 5 minutes at RT and the sections were re-fixed in 4% paraformaldeyde (PFA)/PBS, for 20 minutes at RT, and briefly washed in PBS (2x5 minutes). 100µl of the probe mix (HM containing 20µl/ml DIG-labeled RNA probe) were added to each slide, covered with a coverslip and hybridization occurred overnight at 65-70°C, depending on the probe (Table 2). The next day, after washing, sections were blocked, with blocking solution (10% fetal bovine serum in TBST) at RT for at least 3 hours and then incubated with anti-DIG antibody coupled to alkaline phosphatase (AP) (Roche), 1:500 in blocking solution, overnight at 4°C, and covered with a coverslip. Additionally, Levamisole hydrochloride (Sigma) was added to the NTMT (0,02g levamisole/25ml of NTMT). The staining of the sections was developed using NBT/BCIP (Roche) in NTMT buffer (1µl/ml NBT, 3.5µl/ml BCIP) and the slides were covered with coverslips. The slides were then mounted with coverslips using Aquatex (Merck).

5. CTGF-EGFP reporter transgenics

5.1 Embryos microinjections

Embryos were generated as previously described and microinjections were performed in 1 to 2-cell stage embryos, always injecting 100 pg per embryo. In a first phase, a pEGFP1-vector containing the promoter of the *ctgf* gene (Fig.5) was injected in the embryos. In a second phase, microinjections were performed following the protocol described in Soroldoni *et al*. 2009([69]), where a pBSII-SK+ vector containing the promoter of the *ctgf*, this time flanked with *I-SceI* sites (Fig.5), was coinjected with *I-SceI* meganuclease (Roche), to increase the rate of genome integration. Both constructs were kindly provided by Dr. Jyh-Yih Chen and the pEGFP1-vector is published in Chiou *et al*. 2006 ([48]).

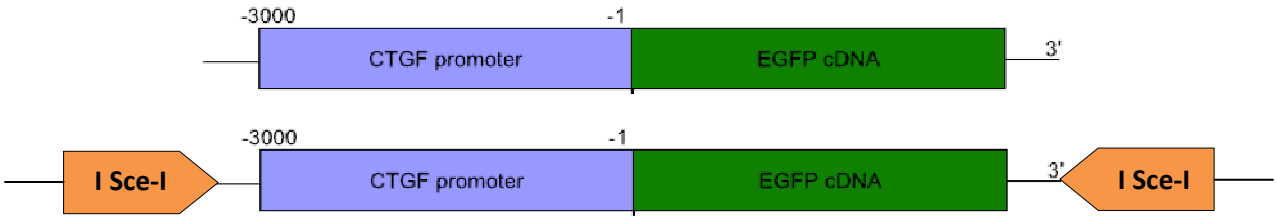

Fig. 5 – Constructs injected in 1 to 2-cell stage embryos. Adapted from *Chiou et al.* 2006([48]).

5.2 Transgenic identification screen

The injected embryos were screened for reporter activity at 24 hpf and the positive ones were selected to grow until adulthood (candidate F0). After three months, candidate F0 was outcrossed with wild-type AB strain adults, and the resulting progeny maintained until 24hpf, the developmental stage where reporter activity was assessed. The embryos were screened to determine which one of the possible founders had incorporated the construct into the germline, being able to transmit the reporter gene to the next generation. The progeny with EGFP expression was then selected to grow until adulthood (F1) and they were kept, as well as the founders (F0), constituting a CTGF-EGFP reporter transgenic population.

6. Microscopy

In situ hybridizations results with fins and embryos were photographed with a Leica Z6APO stereoscope equipped with a Leica DFC490 digital camera. For *in situ* hybridizations in longitudinal sections, pictures were taken using a Leica DM2500 optical microscope equipped with a Leica DFC420 digital camera. The embryos were screened for reporter activity using a Leica MZ16F fluorescence stereoscope and the transgenic embryos were photographed on a Zeiss LSM 5 Live confocal microscope.

Results

1. Characterization of the expression of Hippo pathway genes during regeneration through *in situ* hybridization

Studies of Hippo pathway gene expression have never been performed in zebrafish regeneration, in a systematic way. Plus, it has never been studied in the adult stage, whether the genes that compose this pathway are expressed. Therefore, to understand whether this pathway has a role in regeneration, it is fundamental to characterize whether the genes of the pathway are being expressed, where this expression is present and whether they contribute for regeneration in the caudal fin. In order to do this characterization, we analyzed, by *in situ* hybridization (ISH), the expression of a selected group of genes intended to be representative of the whole pathway. This group of genes included *nf2b* (homolog of the mammalian upstream component *nf2*), *stk3* and *sav1* (homologs of the mammalian core components *mst1/2* and *sav*), *yap1* (homolog of the mammalian transcription coactivator *yap*), *birc5a* and *ctgf* (homologs of the mammalian downstream components *birc5* and *ctgf*) and *frmd6* (homolog of *Drosophila* target gene/upstream regulator *ex*) (Fig.6).

In zebrafish regenerating caudal fin, we found that *nf2b* seems to be weakly expressed at 24 hours post-amputation (hpa) in the blastema (Fig.6A), while at 48hpa the expression is observed in the distal part of the blastema (Fig.6B). This expression domain is maintained until 72hpa (Fig.6C), although the signal is weaker, and it does not seem to be detected in later regeneration stages (Fig.6D-E). However, our results were not consistent for this gene. Regarding *stk3,* we observe that this gene is strongly expressed at 24hpa in the blastema and it seems to be weakly expressed throughout the fin also (Fig.6G). Through longitudinal sections of caudal fins at this stage, we confirmed that *stk3* expression is distributed in the several tissues of the fin, but it is especially increased in the blastema (Fig.6K'). From 48hpa onwards, the expression becomes restricted to the distal part of the blastema and this is maintained until 72hpa (Fig.6H-I). It was not observed expression for this gene at later stages (Fig.6J-K). A similar pattern was observed for *sav1*, since its expression is present in the blastema at 24hpa (Fig.6M), in the distal blastema at 48 and 72hpa (Fig.6N-O), and it is not detected after 72hpa (Fig.6P-Q). In the case of *yap1*, this gene shows high levels of expression within the blastema at 24hpa (Fig. 6S). This was corroborated in longitudinal sections at the same stage, that show a very strong and restricted domain of *yap1* expression, in the blastema (Fig.6L'). Regarding the remaining tissues, it was not clear from our results, whether there is a weak expression or background staining. The expression domain of *yap1* gets confined to the distal blastema at 48hpa and it is maintained until 120hpa (Fig.6T-W), becoming even more distally restricted over time. The

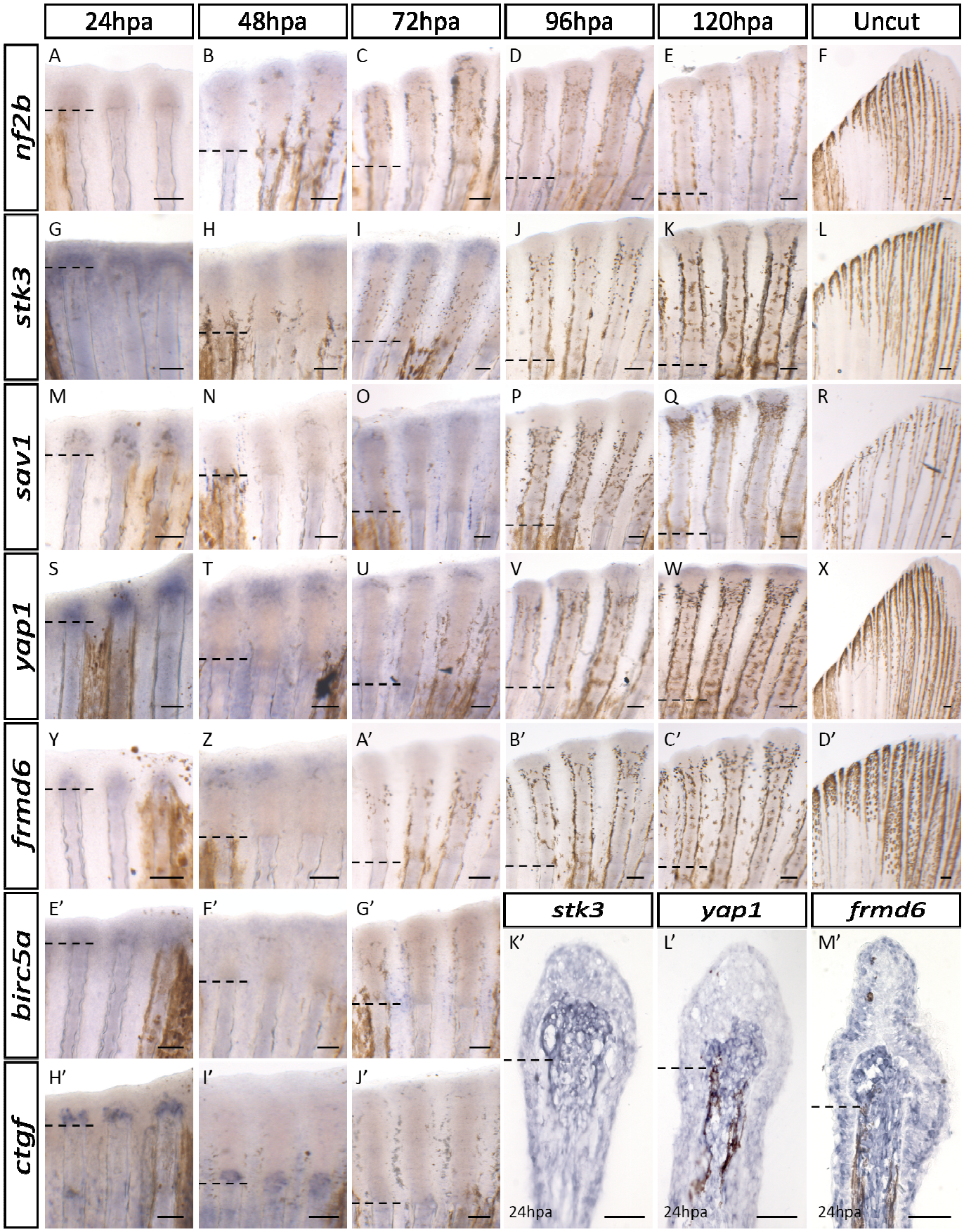

Figure 6 – Expression pattern of Hippo pathway genes during five stages of caudal fin regeneration. (A-J') Whole-mount *in situ* hybridization of (A-F) *nf2b*, (G-L) *stk3*, (M-R) *sav1*, (S-X) *yap1*, (Y-D') *frmd6*, at 24, 48, 72, 96, 120hpa and uncut fins and of (E'-G') *birc5a* and (H'-J') *ctgf* at 24, 48, 72hpa fins in the distal region. (K'-M') *In situ* hybridization in longitudinal sections of caudal fins of *stk3* (K'), *yap1* (L') and *frmd6* (M') at 24hpa. Dashed lines indicate the amputation plane. Scale bars:100µm in whole-mounts and 50µm in sections.

uncut samples were used as negative controls, because if we see transcription activation of the analyzed genes in the amputated fins, when compared to undamaged fins, it indicates that these genes may be important for regeneration. As expected, the expression of the previously described genes was always undetectable in the uncut fins (Fig.6F,L,R,X).

After knowing that the core components of the Hippo pathway were expressed during regeneration, we next wanted to find whether a target gene of YAP1 is expressed, which then could be used as readout of the pathway. In *Drosophila,* one of the upstream components which activates the core kinase cascade, *expanded (ex)*, is also one of the most important target genes of YAP1. Hence, we analyzed the expression pattern of *frmd6,* the zebrafish ortholog of *ex,* and we found that it is expressed in the blastema at 24hpa until 72hpa (Fig. 6Y-C'), thus having a similar expression domain as the other genes already described above. Additionally, longitudinal sections show that *frmd6* is not only present in the blastema but it seems to be also expressed in the outer epidermis at 24hpa, except for the basal epidermal layer (Fig. 6M'). Regarding the uncut fin, expression of *frmd6* was never detected (Fig.6D'). Moreover, two other possible target genes, the anti-apoptotic gene *birc5a* (Fig.6E'-G'), which is one of the two homologs of birc5 in zebrafish, and the cell-growth inducer *ctgf* (Fig.6H'-J'), were also studied. Both genes seem to be expressed in the blastema at the amputation site at 24hpa (Fig.6E' and H'), although the results for these two cases were not consistent.

In addition, we also analyzed *four-jointed,* whose homolog encodes a kinase involved in the upstream regulation of the pathway in *Drosophila,* and *birc5b*, the second zebrafish homolog of *birc5*, but expression of these genes was never observed during regeneration. This might result from low expression levels which are not detected with the probes or because our probes did not work (data not shown).

Together, our data show that several important genes of the Hippo pathway are upregulated during caudal fin regeneration at 24hpa and that this upregulation may last until 72hpa or later, in the case of *yap1*. This suggests that the Hippo pathway is conserved in zebrafish and possibly involved in the regulation fin regeneration. In particular, it also indicates that *frmd6* might be conserved as a target gene of YAP1 in zebrafish, since its expression is upregulated in the regenerating fin. In order to take more conclusions about the activity of the pathway, a further analysis is necessary and it is fundamental to confirm whether the candidate target genes are actually being activated through the transcriptional coactivator YAP1.

2. Expression study of Hippo pathway genes during regeneration through RT-PCR

To corroborate the results obtained with ISH, the previously selected genes were studied by semi-quantitative RT-PCR. Amplification of *β-actin* was used as a positive control to ensure the reliability of the RT-PCR[65], since it is known to be ubiquitously expressed in the fin, regardless of regeneration. On the other hand, *cardiac myosin light chain 2* (*cmlc2*) was used as a negative control because it is expressed solely in the heart[58]. Additionally, 5dpf embryos cDNA was also used as a positive control because all of our tested genes were cloned from cDNA extracted at this stage, so we knew that they are expressed.

Here we provide evidence that confirm our ISH results, showing that several genes of the pathway are being expressed during fin regeneration, until 72hpa (Fig.7). For some cases such as *stk3*, *yap1* and *frmd6* (Fig.7A and B), the RT-PCR results show expression of these genes during the three first days of regeneration, although the bands are weak, especially at 24hpa. Regarding *stk3* and *yap1,* this might be explained by the lower number of amplification cycles. In the case of *nf2b* and *ctgf-c20* (Fig.7B), the expression seems clear comparing to that obtained in ISH, however, it is important to note that these results are preliminary, so it is necessary to reproduce them. It is noteworthy that all these genes show an apparent increase in the expression over time, when considering these regeneration stages, with the exception of *yap1* at 72hpa (Fig.7A). Moreover, it is also demonstrated that all these genes are expressed in the system during homeostasis, since they are detected in uncut fins.

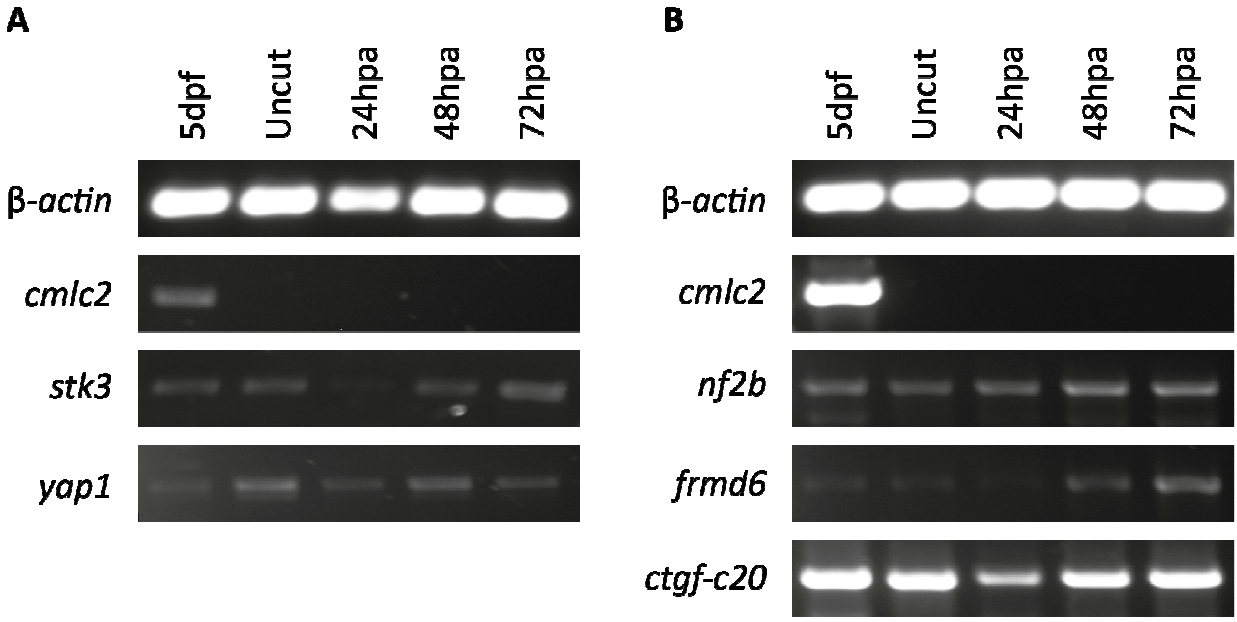

Figure 7 - Semi- quantitative RT-PCR was performed on selected genes using cDNA of uninjured and regenerating fin-tissue isolated at 24, 48, and 72 hpa as template. In addition, cDNA of 5dpf embryos was also used as a positive control. The genes were amplified for 25 cycles (A) or 35 cycles (B).

The expression of other genes such as *birc5a, four-jointed* and *sav1* was also assessed by RT-PCR but we were unable to detect their expression during fin regeneration. However, it has already been shown that *birc5/survivin* is expressed during regeneration from 24hpa until 72hpa, showing an expression peak at 48hpa, and also in uncut fins[65,70].

Therefore, this assay demonstrated that some relevant genes of the pathway such as *nf2b*, *stk3*, *yap1*, *frmd6* and the putative target gene *ctgf-c20* are expressed throughout the first days of regeneration, even though some results are very preliminary. Moreover, it confirmed some of the previous results obtained through ISH and simultaneously provided new evidence on other genes whose expression was not clearly detected using ISH, further supporting the hypothesis that the Hippo pathway is conserved in zebrafish and involved in the regulation of caudal fin regeneration.

3. Establishment and preliminary characterization of a CTGF-EGFP reporter transgenic line

One of the main advantages of working with zebrafish, is the possibility to create transgenics. This is a fundamental tool of this model, which can be used to visualize *in vivo* the expression of a specific gene. In this project, we established a CTGF-EGFP reporter transgenic line, where the promoter of *ctgf* drives the expression of an enhanced form of GFP. The *ctgf* gene, one of *yap1* putative target genes, is responsible to encode a secreted protein, member of the CCN family, which is important for vertebrate development. This transgenic line might be particularly important, because if *ctgf* is confirmed as a target gene, it will work as an *in vivo* readout of the pathway output.

During the establishment of this line, the founder fish was outcrossed five times, in order to determine the percentage of germline transmission. In average, the five clutches generated 22 embryos expressing EGFP, in a total of 112,6 embryos, which means 19,5% of the total sample (Table 3).

	Dead	$EGFP^-$	$EGFP^+$	Total
Cross 1	18	26	12	56
Cross 2	11	50	16	77
Cross 3	18	114	25	157
Cross 4	66	112	40	218
Cross 5	9	29	17	55
Average (%)	**24,4 (21,7%)**	**66,2 (58,8%)**	**22 (19,5%)**	**112,6 (100%)**

Table 3 – Number of embryos that resulted from the outcross of the CTGF-EGFP founder with wild-type, discriminating those that died prematurely (Dead) and those that showed ($EGFP^+$) or did not show ($EGFP^-$) EGFP activity.

The EGFP-positive embryos were analyzed during the initial developmental stages to determine which structures showed reporter activity, to compare with the results described by Chiou *et al*. 2006([48]), who provided us with the reporter construct. We found that at 24hpf EGFP was strongly detected in the notochord, head epidermis and other epithelial cells covering the yolk (Fig.8A). These structures maintain EGFP activity until 72hpa (Fig.8B and C). In later stages, from 48hpf until 72hpf, EGFP was also present in the heart, the pectoral fin buds and some cells of the fin fold (Fig.8B and C).

Figure 8 – Expression pattern of the CTGF-EGFP transgene at different developmental stages (A-C). Embryos staged at 24hpf (A and A'), 48hpf (B) and 72hpf (C) have been characterized. (A') Same embryo represented in A, in bright field. Expression is observed in the head epidermis (A), notochord (A,empty arrowhead) and epithelial cells covering the yolk (A,full arrowhead) at 24hpf. At later stages (B,C), expression is still detected in the epidermis and notochord (empty arrows), but also in the heart (full arrowheads), pectoral fin buds (full arrows) and some cells in the fin fold (empty arrowheads). Scale bars:500 µm for all figures.

These results are consistent to what has been reported by Chiou *et al.* 2006([48]), although we found some differences such as the stronger expression of EGFP throughout the head epidermis and the EGFP detection in the heart, pectoral fins and cells in the fin fold, not previously reported. However, it is important to point out that their results are based on the observation of injected embryos, which often show transient and highly mosaic expression of the reporter.

Following the preliminary characterization of our transgenic line, we performed an ISH to assess whether the *ctgf* RNA expression recapitulates EGFP reporter expression, which was important to validate our transgenic line. Here we show that at 24hpf, *ctgf* is being upregulated along the midline of the embryo, namely in the floor plate and the adaxial cells of the somites (Fig.9A and A'). At 48hpf, expression becomes restricted to the notochord, pectoral fin buds, heart and presumably the developing ethmoid in the head (Fig.9B and B'), and it maintains the

same pattern until 72hpf (Fig.9C and C'). These results corroborate what has been previously reported for one of the two paralogs for this gene in zebrafish, *ctgf-c20*[49], although we see expression along the full notochord at 48hpf. A more detailed analysis, including sections of the embryos, would be important to clarify the stained structures. Thus, these results are consistent with the EGFP reporter expression observations from the transgenic line we have established, showing *ctgf* expression in the same main structures (head, notochord, heart and pectoral fin buds), which is important to validate our transgenic.

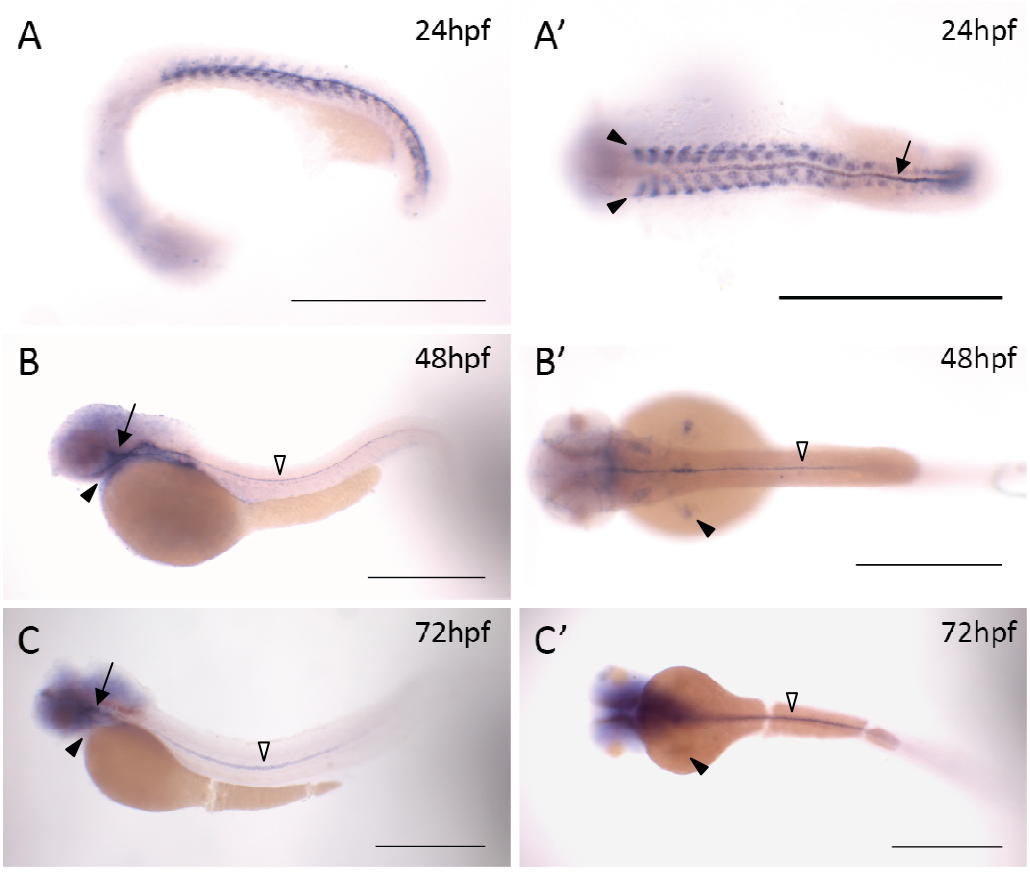

Figure 9 - Expression pattern of *ctgf* detected by *in situ* hybridization in embryos staged at 24hpf (A-A'), 48hpf (B-B') and 72hpf (C-C'). (A-C) Lateral view and (A'-C') Dorsal view. Expression is observed in adaxial cells (A',full arrowheads) and floor plate (A',arrow) at 24hpf . At later stages expression is detected along the notochord (B-C',empty arrowheads), heart (B,C,fullarrowheads), ethmoid plate (B,C,arrows) and pectoral fin buds (B',C',full arrowheads). Scale bars:500 µm for all figures.

4. Expression study of JNK pathway genes during regeneration

To explore the recently reported connection between JNK and Hippo pathways[62], we decided to study the gene expression of two genes that compose the JNK pathway, *junb* and *c-jun,* that are activated upon fin amputation[61]. Through ISH in the zebrafish regenerating caudal fin, we analyzed systematically the expression pattern of *junb* and demonstrated that this gene is

upregulated throughout the wound epidermis, blastema and inter-ray space at 24hpa (Fig.10A). This is a similar pattern to what was described at 12hpa[61]. At 48hpa we observe that the *junb* transcript is almost restricted to the wound epidermis (Fig.10B), thereby confirming what has been reported at this stage[71]. In order to perform a more complete analysis, we analyzed the expression of this gene after 48hpa to see whether the pattern is maintained and what we saw is that *junb* seems to be present exclusively in the epidermis until 120hpa and this domain gets narrower over time (Fig.10C-E). In the uncut control fins, expression of *junb* is undetectable (Fig.10F). ISH for this gene was also performed in longitudinal sections of caudal fins, but the results were not conclusive (data not shown). In the case of *c-jun,* it has been described that its expression is detected in the first 12hpa and afterwards it is downregulated. However, we were not able to reproduce the result at 12hpa nor to detect expression before that early stage of regeneration (data not shown). These results show that *junb*, a gene of the JNK pathway, is upregulated in the regenerating fin from 24 to 120hpa and that the expression domain is distributed throughout the fin at 24hpa, becoming restricted to the epidermis from 48hpa and onwards.

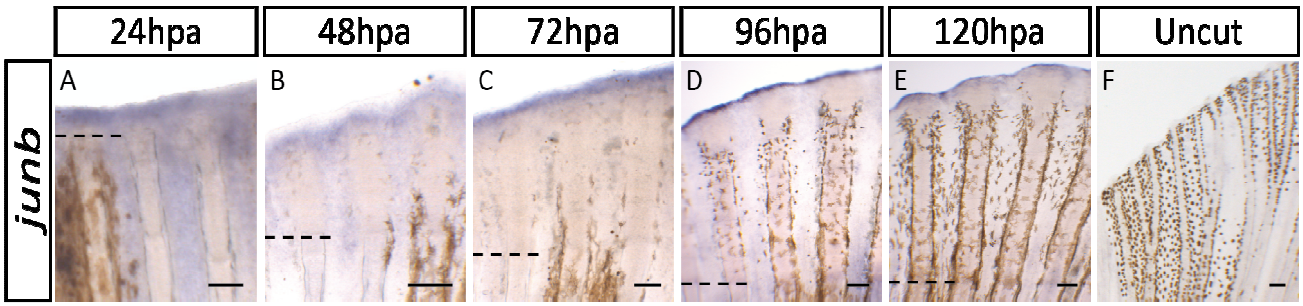

Figure 10 - Expression pattern of *junb* during five stages of caudal fin regeneration. (A-F) Whole-mount *in situ* hybridization at 24,48,72, 96,120hpa and uncut fins in the distal region. Dashed lines indicate the amputation plane. Scale bars:100µm for all figures.

Discussion

Growth is a fundamental and universal process in development, however, despite its importance, the mechanisms that ensure the right proportions of an organ (and an organism) remain poorly understood. Therefore, understanding growth control remains one of the biggest challenges in Developmental Biology. This may result from the fact that size control is a highly complex process involving different mechanisms that act in a coordinated manner in order to achieve the proper size. The recently discovered Hippo pathway is providing new insights on this topic, since it has revealed to be a potent regulator of tissue size, modulating cell proliferation and apoptosis in several organisms. When this pathway is deregulated, dramatic tissue overgrowth occurs, which makes this pathway a promising candidate to be one of the major mechanisms of growth control. One of the classical questions on the field of Regenerative Biology is how the zebrafish fin is able to restore the lost tissues upon amputation, maintaining always the same original size, regardless of the number of amputations. This must be a tightly regulated process because regeneration is characterized by a big increase in the proliferation rate during a short period of time and it never gets deregulated leading to a possible tumor formation. Thus, the Hippo pathway may conserve the same functions in zebrafish and therefore emerges as a suitable candidate to play a role in the regulation of the fin size during regeneration. This pathway has been extensively studied in *Drosophila* and mammals, although less is known about its functionality in zebrafish.

The main goal of this project was to determine whether the Hippo pathway works as a growth regulator in zebrafish caudal fin regeneration. In order to characterize the pathway in this system, we performed gene expression studies through *In situ* hybridization (ISH) and semi-quantitative Reverse Transcription-PCR (RT-PCR) on a selected group of Hippo pathway genes in several stages of caudal fin regeneration. We show that several relevant genes are being upregulated during regeneration. From the upstream components, we found that *nf2b* seems to be expressed at 24hpa until 72hpa, in both assays, although the results were not consistently repeated, so this gene needs further study. Through ISH, the tumor suppressor genes *stk3* and *sav1,* which encode two core components of the pathway, are shown to be expressed in blastema at 24hpa. From 48hpa until 72hpa the expression domain becomes restricted to the distal part of the blastema, not being detected at later stages. In particular, *stk3* seems to be weakly expressed throughout the fin at 24hpa. To assess more accurately the localization of the expression domain in the fin, we performed ISH in longitudinal sections and we observed that at 24hpa, the expression was consistent with the whole-mount result, with a stronger expression in the blastema and a weak expression with a widespread distribution. The RT-PCR results corroborated, for *stk3*, that its expression is present from 24hpa until 72hpa, although we have

not analyzed later stages with this assay. Regarding *sav1*, the RT-PCR did not work, since no expression was detected in the 5dpf control, so it requires further work. In the case of the *yap1,* which encodes the transcriptional coactivator of the pathway, the expression is strongly observed at 24hpa at the blastema and is maintained until 120hpa. From 48hpa and beyond, the gene is only expressed in the distal part of the blastema, and it becomes more distally restricted over time. An analysis to a longitudinal section at 24hpa, showed a clear expression domain restricted to the blastema, consistent with the whole-mount result. It is not clear from our results, whether the remaining tissues show weak expression or just background staining.

In order to find a target gene that could work as a readout of YAP1 activity we analyzed the expression of three putative targets: *birc5a*, *ctgf* and *frmd6.* The ISH analysis was not consistent between different experiments for *birc5a* and *ctgf,* preventing further conclusions, although they seem to show expression in the blastema at 24hpa. The RT-PCR showed expression for *ctgf* in the three analyzed stages of regeneration, although this is very preliminary. The RT-PCR did not work for *birc5a*, since we did not detected expression in the 5dpf control, although it is described that this gene is expressed at 24, 48 and 72hpa. Therefore, the analysis should be repeated for both genes and needs further study. In the case of *frmd6,* we show, through ISH and RT-PCR, that this gene is weakly expressed in the blastema at 24hpa, maintaining expression until 72hpa. On the other hand, when we observed a longitudinal section at 24hpa, the expression is strong in the blastema and the outer epidermis, which shows another domain of expression not observed in the whole-mount result. This may result from the fact that in sections the tissue is more available to the probe than whole-mount tissues, but the result of ISH in sections was not consistently obtained in independent experiments, so we have to repeat the ISH study for this gene. If it is confirmed this may reflect that YAP1 is activating transcription at 24hpa in blastema, which is expected, but also in the outer epidermis, which is quite surprising, since the proliferation is mainly happening in the blastema during regeneration. It is important to note that even if the expression of these putative target genes is shown, further studies are necessary, such as biochemical studies, to establish them as YAP1 target genes. Ideally, the ISH analysis should be performed in tissue sections for all the selected genes, in the different regenerating stages, to confirm the expression domains we reported in the whole-mount ISH, however, we were unable to do so, due to time constraints. Regarding the uncut fins, the ISH showed no expression, which means that the genes are being upregulated in regeneration, when compared to undamaged fins. However, through RT-PCR, which is a more sensitive technique, we show that all the genes seem to be expressed in the uncut fin. Our RT-PCR analysis is only semi-quantitative, but this expression seems weak (except for *ctgf* that needs confirmation), which may reflect just a basal gene expression.

All together, these results show that several genes of the pathway are being upregulated

during zebrafish caudal fin regeneration, most of them until 72hpa and *yap1* until 120hpa, suggesting that the pathway might be important to regulate the regeneration process and thereby contributing for the maintenance of the fin original size. It is important to note that neither of the used techniques is quantitative so, in order to have a precise idea of the expression levels in the different genes and regeneration stages, it is necessary to perform a quantitative PCR in the future.

After the expression studies through ISH and RT-PCR, taking advantage of one the zebrafish main features, we established a transgenic line that allows to visualize in *vivo* the expression pattern of *ctgf,* a putative target of YAP1. In particular, in this CTGF-EGFP line, the promoter of *ctgf* drives the expression of an enhanced form of GFP. Due to the fact that establishing a transgenic line is a highly time-consuming process, it was not possible, during this project, to do more than a preliminary characterization of this line in the first developmental stages (24, 48 and 72hpf). This characterization was important to compare with the reported results for the expression in the early stages of development using the same construct[48]. Here we show that our reporter transgenic line is characterized by a strong expression in the notochord and the head epidermis at 24hpf. Also some epithelial cells in the surface of the yolk showed EGFP reporter activity. On the other hand, through ISH, it seems that *ctgf* expression is strongly detected at 24hpf only in the floor plate and the adaxial cells in the somites. To clarify these apparently different patterns, sections of the embryos could be more informative. At 48hpf and 72hpf *ctgf* expression is detected, in the ISH and the transgenic line, in the notochord and the head epidermis but also other structures such as the heart and the pectoral fin buds. Additionally, in the transgenic line also some cells in the fin fold show EGFP activity at these stages. It is noteworthy that our results are based on observations of a first generation (F1), which might result in some non specific signal and therefore may explain the few differences between the two analysis. The expression in the notochord and the head is consistent with what has been previously reported[48], but we found other structures that have not been described as having EGFP expression. This may be due to the fact that the published results about this construct activity were based in the observation of injected embryos, which are highly mosaic and usually show transient expression of the reporter. Additionally, we determined that our transgenic line transmits the transgene to 19,5% of the offspring and one explanation for this lower percentage value, comparing with the expected Mendelian frequency (50%), is the high mosaicism of the germline of the injected fish (F0). Therefore, in this project we established and characterized during the first three days of development a CTGF-EGFP transgenic reporter line. Moreover, this characterization together with our ISH analysis recapitulate, in most of the structures, the expression already described for this gene, thereby providing good indications to validate the specificity of our transgenic line. In addition, to validate our transgenic, we can do

an ISH for *ctgf* in transgenic embryos to assess co-localization between expression patterns and an immunohistochemistry study using an antibody against *ctgf* to assess co-localization between the gene expression and the protein activity. This transgenic line might be particularly important because, if *ctgf* is confirmed as a target gene of YAP1, it can be used as an *in vivo* readout of the pathway activity, creating several possibilities for the study of the Hippo pathway. In particular, we are interested to see with this transgenic line whether whether there is an upregulation of *ctgf* in the fin, upon amputation, also in the adult stage.

Finally, since the JNK pathway is important for regeneration in zebrafish[61] and due to the recently demonstrated interaction between JNK and Hippo pathways in *Drosophila*[62] repair mechanisms, we decided to explore this connection. Through ISH, we analyzed the expression of *junb*, a JNK pathway gene, and we found that expression is detected in the caudal fin during regeneration in the 24hpa blastema and inter-ray space. At 48hpa, the expression domain seems to become restricted to the wound epidermis, where it is maintained until 120hpa, becoming more distally restricted over time. This was the first time that a broad and systematic analysis was performed for this gene during regeneration. *c-jun* is reported to be downregulated at 12hpa, however, we were unable to reproduce such result with our probe. This issue requires further characterization but it would be important to determine if JNK pathway interacts with Hippo, since it would support a role for the Hippo pathway in regeneration and a means of Hippo regulation via JNK. For instance, it would be interesting to inactivate or overexpress JNK and assess whether there is an impact in the Hippo pathway, specifically whether it affects YAP1 activity in the regenerating fin and subsequently the proliferation.

Collectively, the results obtained with this project suggest that the Hippo pathway is conserved in zebrafish and that it may play an important role in the regeneration process of the caudal fin, since most of the tested components are upregulated in the regenerating tissue. Moreover, this work has helped to get a better understanding about how the Hippo pathway works during fin regeneration, although further studies are necessary to establish this pathway as one of the mechanisms that regulate growth in this process. In the future, it is important to perform biochemical studies in order to establish the interactions between the components of the pathway and to establish targets of this pathway in zebrafish. Furthermore, it is fundamental to perform a systematic characterization at the protein level. This kind of analysis is in progress in our lab, through immunohistochemistry studies, however, it is still premature to take conclusions. Additionally, we are planning to perform ISH together with immunohistochemistry to evaluate co-localization between gene expression and proteins. Therefore, if the Hippo pathway is confirmed as a major mechanism of growth control in the fin regeneration, it may help us understanding how the fin recovers always the same original size after amputation, one of the classical questions in the Regenerative Biology.

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